

## Meristem-Localized Inducible Expression of a UDP-Glycosyltransferase Gene Is Essential for Growth and Development in Pea and Alfalfa

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*PsUGT1*, which encodes a microsomal UDP-glucuronosyltransferase, was cloned from root tips of *Pisum sativum*. *PsUGT1* expression is correlated with mitosis and strongly induced in dividing cells. A region at the C terminus of the encoded protein is closely related to the UDP-glucuronic acid binding site consensus sequence, and the protein encoded by *PsUGT1* catalyzes conjugation of UDP-glucuronic acid to an unknown compound. Overexpression of *PsUGT1* sense mRNA has no detectable effect on transgenic pea hairy root cultures or regenerated alfalfa. However, inhibiting *PsUGT1* expression by the constitutive expression of antisense mRNA (under the control of the cauliflower mosaic virus 35S promoter) markedly retards growth and development of transgenic alfalfa. Cell structure and organization in the antisense plants are similar to those of controls, but plant growth is reduced and development is delayed. This inhibition in growth is correlated with a twofold delay in the time required for completion of a cell cycle and with a >99% inhibition of border cell production. Inhibition of *PsUGT1* expression by meristem-localized inducible expression of *PsUGT1* antisense mRNA (under the control of its own promoter) is lethal both in pea hairy roots and in transgenic alfalfa plants. These results indicate that *PsUGT1* expression is required for normal plant growth and development, and they are consistent with the hypothesis that this UDP-glycosyltransferase regulates activity of a ligand(s) needed for cell division.

### INTRODUCTION

Much of what is understood about mitosis in plants is based on extrapolation from how cell division functions in other systems, such as yeast (Scheres and Benfey, 1999). Identification of new genes that control this multicellular process in plants is hampered by the lack of a system in which cell division can be controlled precisely and synchronized among individuals. Recent studies have made it possible to synchronize cell division in root cap meristems by the experimental manipulation of root cap development (Brigham et al., 1998). Root caps provide a well-defined, easily accessible system in which to study aspects of cell structure and function, including mitosis (Feldman, 1984; Sievers and Hensel, 1991). Root caps, which are attached to root apices, protect the apical meristem, serve as gravisensing tissue, and may facilitate penetration of growing roots through the soil (Sievers and Hensel, 1991). Gravity-sensing statocytes develop from meristematic cells and are transformed into secretory cells, which produce mucilage before they finally reach the periphery of the root cap. The terminal step in root

cap development is the complete separation of peripheral root cap cells as they differentiate into border cells (Brigham et al., 1995, 1998). Populations of detached border cells constitute a uniquely differentiated component of the root system that functions to influence the ecology of the rhizosphere (Hawes et al., 1998).

Under controlled laboratory conditions, the process of root cap development in cereals, legumes, and many other species is tightly regulated. Border cells control root cap development by releasing an extracellular signal that suppresses mitosis in the root cap meristem (Brigham et al., 1998). Thus, when a species-specific maximum number of border cells (ranging from a few dozen for tobacco to >10,000 for cotton and pine) accumulates outside the root cap periphery of seedlings maintained for 24 hr on damp filter paper, root cap turnover and border cell production cease (Hawes and Lin, 1990). In pea, alfalfa, maize, and other species in which it has been examined, the process can be experimentally induced and synchronized from plant to plant. This is accomplished by removing the existing border cells, either by immersing the root tip in water or by gently wiping the cells from the root tip surface (Hawes and Lin, 1990; Brigham et al., 1995, 1998). The cellular consequences of removing border cells have been examined in

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detail, using pea as a model system. Within 5 min, increased mitosis in the root cap meristem of pea can be measured by counting increased numbers of mitotic figures; within 30 min, a ninefold increase in mitosis is reached. New border cells separating from the cap periphery can be collected beginning in <1 hr. After 2 hr, presumably after a new set of border cells has been formed (all of which separate from the cap periphery within 24 hr), the number of cells undergoing mitosis in the root cap meristem returns to preinduction levels.

We exploited this system to isolate mRNAs whose expression is correlated with cell division and differentiation in root caps of pea (Woo and Hawes, 1997). Of the four genes characterized, three of them encoded proteins whose predicted functions were not surprising given their correlation with cell division. One was similar to hydroxyproline-rich glycoproteins, another to an alfalfa callus protein and the mouse tumor protein P23, and the third to a highly basic 60S ribosomal protein, L41. We show here that a fourth gene, *PsUGT1*, encodes a UDP-glucuronosyltransferase (UGT) whose expression in meristematic cells is induced in correlation with mitosis and is required for normal development of transgenic pea hairy roots and alfalfa plants. Our results are consistent with the hypothesis that *PsUGT1* is involved in glycosylation of an unknown compound(s) that plays an essential role in plant growth and development.

## RESULTS

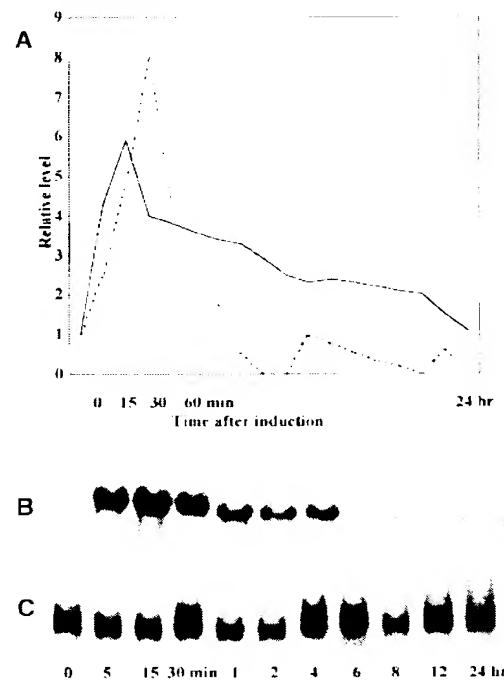
### Cloning of *PsUGT1* mRNA and Induction of Its Expression during Root Cap Cell Differentiation

To study gene expression related to cell division and differentiation in root caps of pea, we exploited the shift in gene expression that occurs when root cap turnover is induced by removing border cells (Brigham et al., 1998). Differentially expressed messages were isolated from a 15-min-induced root tip cDNA library by comparing mRNAs from uninduced and 15-min-induced roots (Woo and Hawes, 1997). After screening  $2 \times 10^5$  clones from an unamplified cDNA library, several messages showed differential expression during the early phase of cell differentiation in root caps (Woo and Hawes, 1997; Brigham et al., 1998).

In this article, expression of one of these genes, designated *PsUGT1* (for *Pisum sativum* UDP-glucuronosyltransferase) based on sequence analysis (below), was examined in detail. A low level of *PsUGT1* expression was detected in uninduced root caps (Figures 1A and 1B, time 0). The amount of *PsUGT1* message increased as quickly as 5 min after induction of root cap turnover and reached a maximum six- to sevenfold increase within 15 min (Figures 1A and 1B). *PsUGT1* message returned to near-basal amounts after 4 to 6 hr. This expression pattern was strongly correlated with mitosis, which reached a maximum

within 30 min after induction of root cap turnover and returned to basal levels after 2 to 4 hr (Figures 1A and 1B).

In leaves, stems, and whole roots, *PsUGT1* was constitutively expressed at the same low level seen in uninduced root tips (data not shown). Whole-mount *in situ* hybridization assays were used to further localize *PsUGT1* expression during root cap differentiation (Woo and Hawes, 1997; Brigham et al., 1998). These assays showed that *PsUGT1* message was barely detectable in uninduced root caps (Figure 2A). The increase in expression that occurred 15 min after induction was localized to the transverse meristem of the root cap (Figure 2B). After 24 hr, when *PsUGT1* message amount had returned to a low level (Figure 1C), expression



**Figure 1.** Developmentally Regulated Expression of *PsUGT1* during Induced Root Cap Development.

**(A)** RNA gel blot analysis. *PsUGT1* message amount increases by six- to sevenfold in 15 min just before a ninefold increase in mitosis. Expression begins to decrease just before mitosis returns to background levels. The solid line represents the relative increase of mRNA amounts in root tips, where the basal amount of mRNA at time 0 is set at 1.0; the dotted line represents the relative level of mitosis in root cap meristems, where the basal level of mitosis at time 0 is set at 1.0 (Brigham et al., 1998).

**(B)** RNA gel blot of poly(A)<sup>+</sup> mRNAs isolated at different time points from induced root tips probed with the *PsUGT1* cDNA.

**(C)** RNA gel blot probed with the *PsUBC4* (Woo et al., 1994) cDNA as a control for equal mRNA loading.



**Figure 2.** Expression of *PsUGT1* in Roots of Pea.

(A) to (F) Whole-mount *in situ* hybridization of roots whose root cap meristems have been induced to undergo cell division by removing border cells from the cap periphery.

(A) Uninduced root tip (time 0) hybridized with the *PsUGT1* antisense cDNA probe.

(B) Induced root tip 15 min after inducing cell division by removing border cells hybridized with the *PsUGT1* antisense cDNA probe. The open arrowhead highlights the increased expression within the root cap meristem.

(C) Induced root tip 24 hr after inducing cell division hybridized with the *PsUGT1* antisense cDNA probe. *PsUGT1* expression within the root cap meristem has returned to preinduction levels and is barely detectable.

(D) Control. Uninduced root tip (time 0) hybridized with the *PsUGT1* sense cDNA probe.

(E) Control. Induced root tip 15 min after inducing cell division by removing border cells hybridized with the *PsUGT1* sense cDNA probe. The open arrowhead highlights the root cap meristem.

(F) Control. Induced root tip 24 hr after inducing cell division hybridized with the *PsUGT1* sense cDNA probe.

(G) *In situ* hybridization in cross-sectioned lateral roots. High expression of *PsUGT1* mRNA can be detected in actively dividing cells of the meristem.

(H) *PsUGT1-uidA* reporter gene expression in transgenic alfalfa. High levels of  $\beta$ -glucuronidase activity can be seen in root tip meristematic tissue within the tap root and an emerging lateral root.

was barely detectable by *in situ* hybridization (Figure 2C). No changes were detectable in control experiments using a *PsUGT1* sense probe (Figures 2D to 2F). *PsUGT1* message also was localized in meristematic tissues of lateral root initials by using *in situ* hybridization, confirming the tissue-specific, developmentally regulated expression of the *PsUGT1* gene in actively dividing cells (Figure 2G). A reporter gene (*uidA* encoding  $\beta$ -glucuronidase) was expressed under the control of *PsUGT1* promoter (−892 to +450). As in untransformed roots, *PsUGT1* expression was localized both in induced root tips and in emerging lateral root initials (Figure 2H).

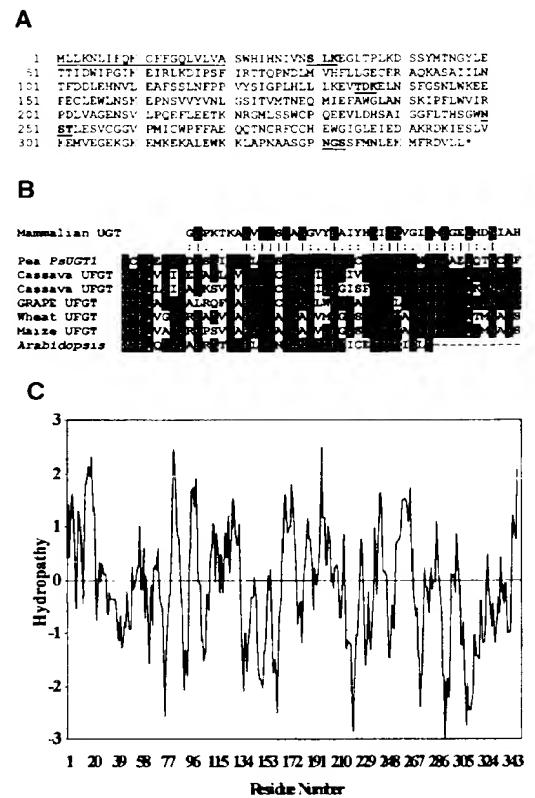
## Structural Analysis of the *PsUGT1* Gene

A *PsUGT1* gene was isolated from a pea genomic library by using the *PsUGT1* cDNA as a probe. Approximately 1 kb of upstream promoter and 2 kb of coding region have been sequenced. The upstream promoter contains TATAAA (−54 to −59) and CCAAT (−92 to −96; numbers refer to the distance from the predicted transcription start site) sequences. No introns were found in the 2-kb coding region. The predicted *PsUGT1* polypeptide has 347 amino acids (Figure 3A) with a putative UDP-glucuronic acid binding site located between amino acids 228 and 277 (Figure 3B) (Mackenzie, 1990). This 50-amino acid region in the encoded protein possesses 38% identity and 81% similarity (if conservative substitutions are considered) with the UDP-glucuronic acid binding site (Figure 3B). A database search revealed several other sequences with similarity in this region. These included flavonol 3-*O*-glucosyltransferases (UDP-glucose:flavonoid 3-*O*-glucosyltransferases) from cassava, wheat (Wise et al., 1990), maize (Furtek et al., 1988), and grape (Spravoli et al., 1994), and UGTs from human (Ritter et al., 1990), rat, and mouse (Iyanagi et al., 1986). *PsUGT1* also possesses potential protein kinase C phosphorylation sites—(S or T)-X-K (residues 30 and 135), where X stands for any amino acid—and two potential N-glycosylation sites N-X-(S or T) (residues 250 and 331) (Figure 3A).

DNA gel blot analysis of pea genomic DNA (data not shown) and extensive rescreening of genomic DNA and pea root tip cDNA libraries suggest that *PsUGT1* is represented by a single gene in the pea genome.

## Confirmation of the Identity of the *PsUGT1* Product by in Vitro Enzyme Activity

Enzyme assays of the in vitro-expressed *PsUGT1* protein were performed to confirm the sequence-based predictions that *PsUGT1* encodes a membrane-localized UGT. Recombinant *PsUGT1* enzyme was generated in *Neurospora crassa* by expressing the *PsUGT1* cDNA under the control of the *N. crassa* *cpc-1* (for cross pathway control 1) promoter.



**Figure 3.** Sequence Analysis of the *PsUGT1*-Encoded Protein.

**(A)** Deduced amino acid sequence of PsUGT1. The putative N-terminal signal peptide in PsUGT1 is underlined. Two potential protein kinase C phosphorylation sites (*(S or T)X-K*, where X stands for any amino acid) and two putative glycosylation sites (NST and NGS) are given in boldface italic. The GenBank accession number is AF034743.

**(B)** Comparison of amino acid sequences with a region of the deduced PsUGT1 together with the conserved UDP-glucuronic acid binding site and corresponding regions in plant UDP-glucose:flavonoid glucosyltransferases (UGT) (lower five sequences). A vertical bar represents an absolute match among all plant sequences with the consensus motif of mammalian UGT sequences. A colon or a period represents conservative substitutions between the conserved mammalian UDP-glucuronic acid binding site and PsUGT1.

(C) Hydropathy plot of the putative PsUGT1 protein. The hydropathy plot was constructed according to Dayhoff (1978) with a window of seven amino acids.

which allows high constitutive expression under the conditions used (Carroll et al., 1994). Both genomic DNA gel blot and RNA gel blot analyses of transformed *N. crassa* confirmed that the *PsUGT1* cDNA was expressed (data not shown). The cellular extracts (including microsomes) from

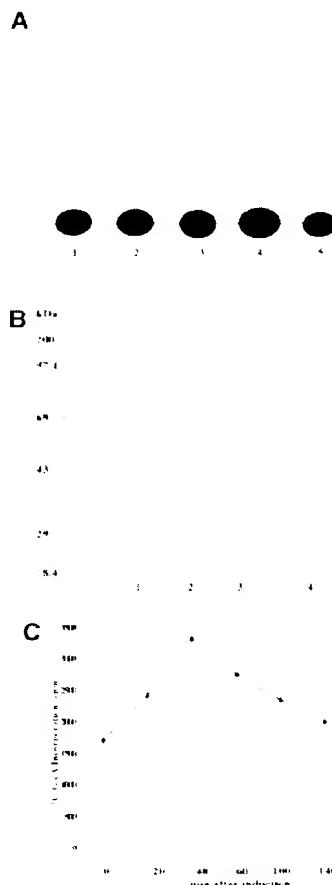
transformed *N. crassa* were used as a source of recombinant PsUGT1 protein. Enzyme assays in which PsUGT1 was incubated with UDP-<sup>14</sup>C-glucuronic acid as a donor and boiled pea tissue extracts as a source of unknown acceptor for the predicted conjugation reaction were performed. The reaction yielded a single <sup>14</sup>C-glucuronic acid-conjugated product (Figure 4A, lane 5). This indicates that PsUGT1 uses UDP-glucuronic acid as a donor to the unknown acceptor compound. In control experiments, conjugated product was not synthesized in response to protein from strains that were not transformed by *PsUGT1* cDNA (Figure 4A, lane 1) or in response to heat-inactivated PsUGT1 (Figure 4A, lane 2). The recombinant protein did not yield a product in response to other UDP sugars, including UDP-glucose and UDP-galactose (Figure 4A, lanes 3 and 4).

#### Localization of PsUGT1 in Microsomes

Like other UGTs, PsUGT1 has hydrophobic amino acids at the N terminus that are characteristic of signal peptides of secretory proteins and most transmembrane proteins (Figure 3A) (Mackenzie, 1990; Bennett and Osteryoung, 1991). The hydropathy profile suggests that PsUGT1 is probably a transmembrane protein with hydrophobic domains (Figure 3C). To localize the *PsUGT1*-encoded protein within pea cells, we transformed pea with a cauliflower mosaic virus 35S promoter-*PsUGT1*-6×His construct to form transgenic hairy roots. The transgenic hairy roots were ground, and the cellular components were size-fractionated by ultracentrifugation into microsomal and cytoplasmic components. Protein was extracted from these components by 6 M guanidine-HCl and size-fractionated by SDS-PAGE. The SDS-PAGE-separated proteins were subjected to protein gel blot analysis using a commercially available antihistidine antibody. This antibody reacted with a protein from microsomal components of 39 to 40 kD (Figure 4B), suggesting that the *PsUGT1*-encoded protein is mainly localized in microsomal membranes.

#### Induction of Endogenous Glucuronosyltransferase Activity during Root Cap Turnover

Tests were conducted to determine whether the expression of the *PsUGT1* message is correlated with an increase in endogenous microsome-bound glucuronosyltransferase activity in root tips induced to undergo mitosis. Microsome-bound glucuronosyltransferase activity in uninduced and induced pea root tips was determined by an in vitro enzyme reaction with UDP-<sup>14</sup>C-glucuronic acid. Endogenous glucuronosyltransferase activity increased within 20 min, reached maximum expression (a twofold increase over uninduced root caps) in 40 to 50 min, and returned to near-basal levels by 140 min after induction (Figure 4C).



**Figure 4.** UGT Activity of the *PsUGT1*-Encoded Protein and of an Endogenous Pea Microsomal Enzyme Whose Levels Are Correlated with *PsUGT1* Expression.

**(A)** In vitro UGT activity of *N. crassa* transformed with *PsUGT1*. Lane 1, untransformed *N. crassa* cellular extracts (control); lane 2, boiled cellular extracts from *N. crassa* transformed with *PsUGT1* cDNA (control); lane 3, cellular extracts from *N. crassa* transformed with *PsUGT1* reacted with UDP-<sup>14</sup>C-galactose; lane 4, UDP-<sup>14</sup>C-glucose; and lane 5, UDP-<sup>14</sup>C-glucuronic acid. Microsomal proteins were incubated with substrate, and UDP-labeled products were separated by thin-layer chromatography and detected by radiography.

**(B)** Protein gel blot of histidine-tagged proteins probed with the antihistidine antibody. Lane 1, cytoplasmic proteins from roots; lane 2, microsomal proteins from roots; lane 3, cytoplasmic proteins from transgenic hairy roots expressing *PsUGT1*; and lane 4, microsomal proteins from transgenic hairy roots expressing *PsUGT1*. An ~40-kD protein from microsomal proteins of transgenic hairy roots was labeled by the antihistidine antibody. Numbers at left indicate protein size standards.

**(C)** Correlation of endogenous glucuronosyltransferase activities with the induction of root cap cell division by the removal of border cells. The glucuronosyltransferase assay was performed three times with three replicate samples for each treatment. The values shown represent the overall means from all three independent experiments. Standard errors were  $\pm 10\%$  of the mean. GlcA, glucuronic acid.

### Meristem-Targeted Inhibition of *PsUGT1* Expression Prevents Hairy Root Development in Pea

Inhibition of *PsUGT1* expression by antisense mRNA mutagenesis was performed in transgenic hairy roots of pea. Inoculation of pea stems with wild-type or vector-only-control *Agrobacterium rhizogenes* resulted in proliferation of hairy roots within 2 weeks (Figure 5A). Transgenic roots were identified using antibiotic selection, and expression of transgenes was confirmed by reverse transcription–polymerase chain reaction and DNA gel blot analysis.

Inhibiting *PsUGT1* in meristematic cells by expressing the antisense mRNA under the control of the *PsUGT1* promoter completely prevented transgenic root development (Figure 5B). The few roots that developed under such conditions did not grow on selective medium because they did not carry the transferred gene. We conclude that this result represents lethality of the expressed gene. This conclusion is based on control experiments in which a known cytolytic gene encoding diphtheria toxin A, whose expression in a given cell results in cell death (Czakó and An, 1991), was placed into *A. rhizogenes* under the control of the 35S promoter. When used to inoculate pea, the resulting lethal phenotype was indistinguishable from that which occurs in response to expression of the *PsUGT1* promoter–*PsUGT1* antisense cDNA construct (data not shown). The discovery that expression of *PsUGT1* antisense cDNA under the control of its own promoter is lethal indicates that the *PsUGT1*-encoded protein plays an essential function in the development of hairy roots of pea. Interestingly, constitutive expression of *PsUGT1* antisense mRNA under the control of the 35S promoter caused no deleterious effects on development (data not shown). Although the 35S promoter is ex-

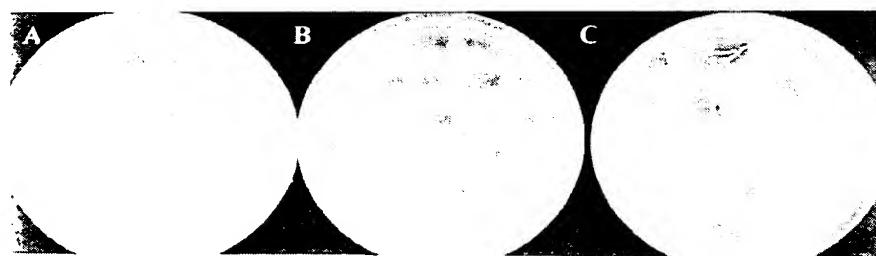
pressed during hairy root development in pea, the cellular localization of its expression varies over time, especially in root tips (Nicoll et al., 1995; Wen et al., 1999). The precise timing and localization of *PsUGT1* expression apparently are central to its biological function such that a change in tissue specificity results in a fundamental change in phenotype.

Overexpression of *PsUGT1* sense cDNA, under the control of the *PsUGT1* promoter or the 35S promoter, caused no detectable effects on hairy root development (Figure 5C). Presumably, the kinetics of the reaction catalyzed by *PsUGT1* are regulated in such a way that excess enzyme has no effect on cellular metabolism.

### Effects of Constitutive and Meristem-Localized Inducible Expression of Antisense *PsUGT1* in Alfalfa

Alfalfa provides a convenient system in which to analyze whole-plant effects of transgene expression because it can be regenerated easily, and, like hairy roots, the transformants can be propagated vegetatively to produce an unlimited number of genetic clones for detailed cellular and physiological analysis. Transformation into alfalfa also made it possible to determine if the *PsUGT1* gene can function in a species other than pea. Thus, alfalfa was transformed with the same sense and antisense constructs used in the experiments described above, and expression of transgenes was confirmed by using reverse transcription–polymerase chain reaction and DNA gel blot analysis.

Expression of a cytolytic toxin gene under the control of the *PsUGT1* promoter was lethal; no regenerants were recovered in dozens of independent transformations, which would normally yield thousands of embryos (data not shown).



**Figure 5.** Effects of *PsUGT1* Antisense cDNA Expression on Pea Hairy Root Development.

**(A)** Wild-type hairy root development. Transformation of a stem explant with *A. rhizogenes* results in proliferation of a dozen or more individual roots from each explant within 10 days to 2 weeks.

**(B)** Lethality of *PsUGT1* promoter–*PsUGT1* antisense cDNA. Inoculation with *A. rhizogenes* carrying *PsUGT1* antisense mRNA under the control of the *PsUGT1* promoter yields almost no hairy roots. The few roots that emerge do not grow on selective medium and do not express the transgene.

**(C)** Overexpression of *PsUGT1*. Normal hairy root development occurs when *PsUGT1* sense mRNA is expressed under the control of *PsUGT1* promoter.

This result confirmed that the *PsUGT1* promoter is functional in alfalfa and illustrated the expected result of a construct whose expression is lethal (data not shown). As in pea hairy roots, overexpression of *PsUGT1* did not cause detectable effects on development, and antisense mutagenesis of *PsUGT1* under its own promoter was lethal; as with expression of the cytolytic toxin gene, no regenerants were recovered among dozens of independent transformations. However, in contrast to its neutral effect on pea hairy roots, constitutive (i.e., 35S promoter-driven) expression of antisense *PsUGT1* cDNA in alfalfa plants resulted in a unique, intermediate phenotype with reduced growth and development, altered morphology, and sterility (Figure 6 and Table 1). One explanation for this discrepancy is possible differences between whole-plant and hairy root development. Alternatively, species-dependent variation in the timing or localization of 35S promoter activity could occur in alfalfa compared with pea, with these differences affecting different *PsUGT1*-mediated processes.

The reproducible production of plants with a measurable phenotype provided a tool with which to examine the way(s) in which *PsUGT1* may act at the cellular level. When compared with vector-only control transformants, the predominant phenotype was a uniform reduction in plant height (Figure 6A). This approximately twofold reduction in plant height was associated with the retention of juvenile leaf morphology (Figures 6B and 6C) and reduced leaf and root mass (Figure 6D). Floral development also was affected in transgenic plants expressing antisense cDNA. Whereas control plants flowered within 8 to 9 weeks, development of the inflorescence in antisense plants occurred only after 13 to 15 weeks (Table 1). In contrast to the long, oval shape of floral buds in control plants (Figure 6E), the morphology of antisense floral buds was short and thick (Figure 6F). Stamens of antisense plants produced much less pollen, and cross-pollination using wild-type pollen on antisense stigmas or reciprocal crosses using antisense pollen on wild-type stigmas yielded no pods (Table 1).

Because plants expressing 35S-*PsUGT1* antisense cDNA are sterile, stem cuttings were used to obtain regenerated clonal transgenic plants for detailed analysis. One explanation for the stunted growth and delayed development of the 35S-*PsUGT1* antisense plants is that cell elongation and/or organization is defective. If so, then alterations in cell shape and organization would be predicted to occur at various stages of development. No differences in cell size and cellular organization were apparent in comparing stem cross-sections (Figures 6G and 6H) or longitudinal sections of control (Figure 6K) and antisense (Figure 6L) plants. Similarly, cellular structure and organization in control (Figure 6I) and antisense (Figure 6J) leaves and roots (data not shown) were indistinguishable from each other. These results are not consistent with the hypothesis that the small size, reduced rate of development, and altered morphology of 35S-*PsUGT1* antisense plants are due to a defect in cell formation and organization. An alternative hypothesis to account

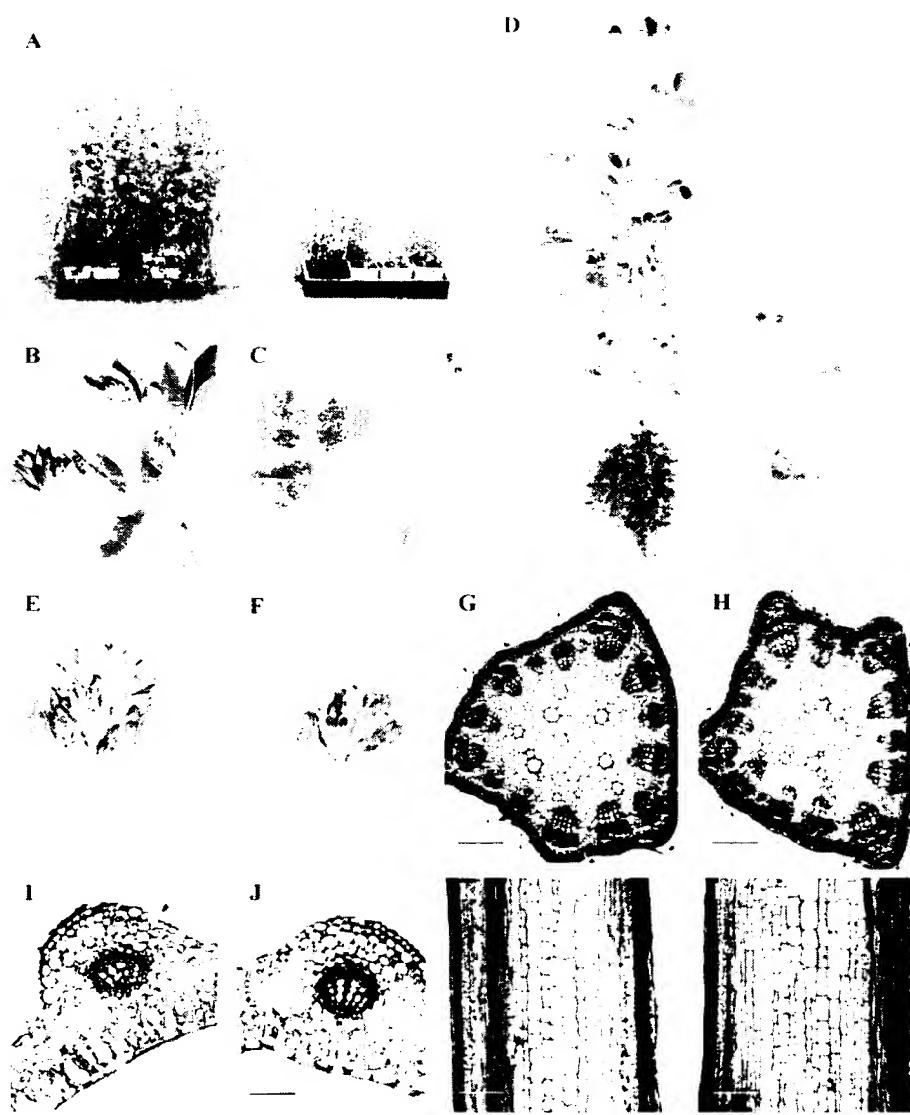
for the observed phenotype is that cell division, rather than cell elongation, is affected. If so, a reduced rate and/or level of DNA synthesis would be predicted to occur in 35S-*PsUGT1* antisense plants as compared with controls.

#### DNA Synthesis in 35S-*PsUGT1* Antisense Plants Is Retarded Compared with Controls

DNA synthesis was measured over time using 5-aminouracil, which synchronizes DNA synthesis during cell division (Jakob and Trosko, 1965). The level of DNA synthesis, as measured by <sup>3</sup>H-thymidine incorporation into synchronized roots, was not significantly different between control and mutant plants. However, the time required to complete a cell cycle was increased by >15 hr in 35S-*PsUGT1* antisense transgenic plants compared with wild-type or vector-only control plants (Figure 7). In roots of control plants, the first peak of DNA synthesis appeared 21 hr after synchronization of cell division, with a second peak occurring at 39 hr. In antisense plants, however, the peaks occurred at 36 and 57 hr. This approximately twofold increase in the time required for the completion of a round of DNA synthesis, which presumably reflects a negative effect on the rate of cell division, correlates well with the approximately twofold reduction in growth observed for whole plants (Table 1). The results are consistent with the hypothesis that *PsUGT1* acts by conjugating a ligand that regulates cell division in plants in such a way that when its level is reduced, cell division is delayed.

#### Border Cell Production Is Inhibited in Alfalfa Plants Expressing 35S-*PsUGT1* Antisense mRNA

Induced expression of *PsUGT1* in root cap meristems is directly correlated with the induced production of root border cells from the root cap. If the expression of *PsUGT1* in fact plays a role in cell division leading to border cell production, then inhibiting *PsUGT1* expression in dividing cells would be predicted to cause a delay in renewed border cell production. This delay would be expected to result in a reduction in the number of new border cells present 24 hr after removing the existing border cells. To test this prediction, we measured border cell production from wild-type plants and 35S-*PsUGT1* antisense plants. As in other tissues, root cap morphology in antisense plants was indistinguishable from that of controls (data not shown). However, renewed border cell production was reduced by >99%, from 2400 ± 340 cells in control plants to <10 cells in 35S-*PsUGT1* antisense plants. Because the experimental plants were genetic clones, no dosage or position effects existed, and little physiological variation occurred among regenerants; the same results were obtained with all of the >70 plants examined. These results are consistent with the hypothesis that altered expression of *PsUGT1* in dividing cells of the root cap during



**Figure 6.** Effects of Constitutive Expression of *PsUGT1* Antisense cDNA (under the Control of the 35S Promoter) in Alfalfa.

(A) Five-month-old control (left) and antisense (right) plants regenerated from stem cuttings. Control alfalfa plants transformed with the pBI101 vector only developed normally (left). Expression of *PsUGT1* antisense cDNA under the control of 35S promoter strongly retarded alfalfa plant growth and development (right).

(B) Long oval-shaped leaves and flowers from control alfalfa plants.

(C) Altered leaf morphology in antisense plants.

(D) One-month-old control (left) and antisense (right) plants regenerated from stem cuttings.

(E) Floral buds from control plant.

(F) Floral buds from antisense plant.

(G) Horizontal cross-section of stem from control plant.

(H) Horizontal cross-section of stem from antisense plant, revealing that its appearance is indistinguishable from that of the control.

(I) Cross-section of control leaf.

(J) Cross-section of antisense leaf, revealing that its appearance is indistinguishable from that of the control.

(K) Longitudinal cross-section of stem from control plant.

the induction of mitosis directly affects the renewed production of border cells by interfering with the activity of a product needed for cell division.

## DISCUSSION

Reversible glycosylation is a universally important mechanism by which certain organisms control the impact of a wide range of hydrophobic compounds that can direct or derange cellular metabolism (Nebert, 1991). In mammals, drug-metabolizing enzymes, such as UGTs, have been proposed to be involved in the regulation of differentiation and morphogenesis as well as detoxification (Nebert, 1991). UGTs are thought to control the steady state levels of ligands, such as steroid hormones, that modulate cell division, growth, and morphogenesis. Steroids together with steroid receptors repress the activity of many transcription factors, such as NF- $\kappa$ B (La Rosa et al., 1994; Ray and Prefontaine, 1994; Caldenhoven et al., 1995), that activate expression of genes involved in cell growth. UGT-mediated regulation of steroid activity by glucuronidation in mammals, then, occurs upstream of signal transduction for the control of cell growth, and mutations that interfere with the UGT-mediated signal transduction pathway result in lethality or early death (Baldwin, 1996). UGTs also facilitate secretion of toxic compounds by increasing their solubility in the cell.

Several thousand glycosylated chemicals have been identified in plants. Glycosylated secondary metabolites include flavonols (Wollenweber and Jay, 1988), anthocyanins (Harborne and Grayer, 1988), monoterpenes (He et al., 1994), and plant hormones (Bandurski et al., 1995). Glycosylation of secondary products invariably results in enhanced water solubility and lower chemical reactivity. Glycosylated compounds are thought of as transportable storage compounds or waste or detoxification products lacking physiological activity. In some cases, xenobiotic compounds are glycosylated by glycosyltransferases (Sandermann et al., 1991). These conjugates can be stored in vacuoles or cell walls for long periods. In the case of phytohormones, including auxin, cytokinin, gibberellin, abscisic acid, jasmonate, and brassinolide, glycosyl conjugates might act as reversible deactivated storage forms. Glycosyl conjugation thus may be important in the regulation of physiologically active hormone levels (Bandurski et al., 1995). In other cases, glycosyl conjugation of plant hormones might accompany or introduce irreversible deactivation.

Despite the obvious potential importance of glycosylated compounds in plant biology, little is known about how their cellular amounts are controlled. Reversible conjugation of regulatory molecules by glycosyltransferases, as occurs in mammals, is a likely possibility. The most studied groups of plant glycosyltransferases are those associated with the biosynthesis of flavonoid glucosides, including flavonol glucosides, flavanone glucosides, and anthocyanins. Although traditional methods for cloning the genes encoding such low-abundance products using cDNA or immunological methods have not been successful, transposable element tagging of the *bronze* (*bz*) locus in maize led to the isolation of a gene encoding UDP-glucose:flavonoid glucosyltransferase, which catalyzes the addition of a glucose moiety to the 3-OH group of a flavonoid, one of the last steps in anthocyanin biosynthesis (Holton and Cornish, 1995).

A new plant glycosyltransferase gene has been characterized in this study. Using the root cap as a model system in which to correlate cell function with gene function (Barlow 1975), we identified several genes whose expression is correlated temporally, with a time window of minutes, and spatially within a few cell layers, with the onset of mitosis. One of these genes, *PsUGT1*, is reported here to encode a UDP glucuronosyltransferase. Within 15 min after removing border cells, mitosis and *PsUGT1* expression increase measurably. Interestingly, a similar 15-min window of mitosis induction has been reported using stage 6 oocytes of *Rana pipiens*, in which the cell cycle can be induced by injection of progesterone (Morrill and Kostellow, 1998). Progesterone is a steroid hormone the active levels of which are controlled by reversible glucuronidation. If a plant signal analogous to progesterone plays a similar role in activating cell division in plants, then the product conjugated by *PsUGT1* would be predicted to exert direct effects on meristematic activity.

Inhibiting the meristem-localized inducible expression of *PsUGT1* by the expression of *PsUGT1* antisense cDNA under the control of its own promoter is lethal. This would be a predictable consequence of inhibiting expression of the only copy of a gene encoding an enzyme needed to regulate cell division. The fact that the pea antisense cDNA was as effective in alfalfa as in pea is consistent with *PsUGT1* being a protein whose structure and/or function is highly conserved among distinct species, as would be expected for an enzyme involved in regulating a process as fundamental as mitosis. Inhibiting expression of *PsUGT1* by antisense cDNA expressed under the control of the constitutive 35S promoter, however, caused no effects on hairy root development. This result would be surprising if the expression of the

**Figure 6.** (continued).

(L) Longitudinal cross-section of stem from antisense plant, revealing that its appearance is indistinguishable from that of the control. Sections in (G) to (L) are stained with toluidine blue-O. Bars in (G) to (L) = 400  $\mu$ m.

**Table 1.** Effects of 35S-*PsUGT1* Antisense cDNA Expression on Alfalfa<sup>a</sup>

Developmental Criteria	Wild-Type Plants	Antisense Plants
Period required for inflorescence formation <sup>b</sup>	8 to 9 weeks	13 to 15 weeks
Fertility	Pods formed 1 week after pollination	Sterile
Morphology of floral buds	Long and oval	Short and thick
Number of floral buds per stem	17 to 22	8 to 12
Amount of pollen on stamen	Abundant	Very little
General morphology of plants	More branches and leaves	Fewer branches and leaves
Growth of lateral branches	Good	Poor
Leaf morphology	Long, oval shape	Juvenile stage
Height at maturity	55 to 65 cm	35 to 45 cm
Rate of DNA synthesis during cell division	Normal	Two times slower than wild type
Border cell production in root tips	Normal	Poor

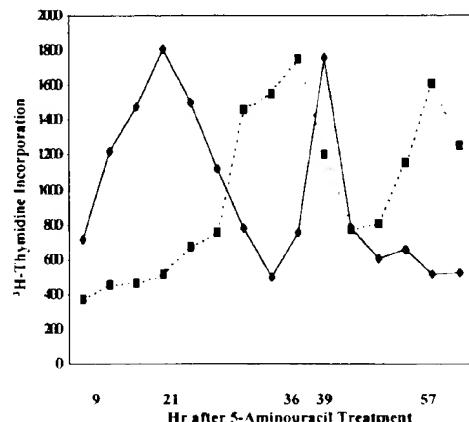
<sup>a</sup> Results shown are measurements of transgenic plants regenerated from third-generation stem cuttings.<sup>b</sup> Time from planting of stem cuttings to formation of the first inflorescence.

35S promoter were active in all tissues all the time. This, of course, is not the case, and in hairy root tips its expression is especially variable according to time and developmental stage (Wen et al., 1999). The absence of an effect in the 35S-*PsUGT1* antisense hairy roots confirms the significance of the rapid, transient, and localized nature of *PsUGT1* expression. Like a gun and a bullet, if the appearance of both

enzyme and substrate does not coincide precisely in time and space, both are perfectly harmless.

We do not know why the same constitutively expressed antisense construct causes substantial problems in the development of transgenic alfalfa, but we suspect that the increased number of mitosis-related events that occur during whole-plant development or, alternatively, species-dependent variation in 35S expression could explain the discrepancy. The fortuitous occurrence of a stable phenotype in the 35S-antisense regenerants has made it possible to demonstrate that a (presumably) "leaky" inhibition of *PsUGT1* expression results in a reduced rate of growth and development. This retarded growth phenotype is correlated with a similarly retarded ability to undergo cell division and to produce root border cells during the normal time frame. The overexpression of *PsUGT1* caused no detectable effects on development of either pea or alfalfa, suggesting that once an adequate amount of enzyme is present in the cell, the reaction catalyzed by *PsUGT1* is regulated by something other than enzyme level.

The results presented here suggest that UGT-mediated glycosylation in plants, as in animals, can play a key role in cellular metabolism. A recent study suggests that the presence of a flavonoid that may be reversibly glycosylated is tightly correlated with cell division in legume root nodules (Mathesius et al., 1998). It will be of interest to determine whether *PsUGT1* expression is correlated with nodule meristem activity as it is correlated with the activity of other root meristems.

**Figure 7.** DNA Synthesis Is Slower in Plants Expressing 35S-*PsUGT1* Antisense cDNA Than It Is in Control Plants.

Cell division in roots was synchronized by treatment with 12 mM 5-aminouracil for 2 hr (Jakob and Trosko, 1965). DNA synthesis in roots of control plants (dashed line) or plants expressing 35S-*PsUGT1* antisense cDNA (solid line) was measured in counts per minute (cpm) by pulse labeling with <sup>3</sup>H-thymidine every 3 hr. The experiment was performed three times, and the values shown are the mean of all three experiments. Standard deviations ranged between 8 and 13% of the mean.

## METHODS

### Plant Growth

Seeds of *Pisum sativum* cv Little Marvel (Royal Seed Company, Kansas City, MO) were surface-sterilized by immersion in 95% (v/v) eth-

anol for 10 min and then 5.25% sodium hypochlorite (full-strength commercial bleach) for 1 hr. During five rinses in sterile distilled water, contaminated seeds (those that floated) were discarded. The remaining seeds were allowed to imbibe sterile water for 6 hr, after which they were placed in Petri dishes containing 1.2% water agar overlaid with sterile germination paper (Anchor Paper Co., Hudson, WI) and incubated in the dark at 24°C. Radicles emerged from the seeds after 24 hr and reached a length of 24 mm in 48 hours. Border cells were removed from the root tips of seedlings when the radicle was 2.5 cm long. Root tips were immersed in 2 to 5 mL of sterile distilled water and agitated to release the border cells (Brigham et al., 1998). The seedlings were placed on fresh sterile germination paper overlaid on water agar. The apical 1-mm segment of each root tip was excised at different times for further analysis.

#### cDNA and Genomic Library Screening

cDNA library construction and screening were as described by Woo and Hawes (1997). A genomic library of *P. sativum* var Alaska was purchased from Clontech (Palo Alto, CA). To identify the *P. sativum* UDP-glucuronosyltransferase (*PsUGT*) gene, we screened 10<sup>6</sup> plaque-forming units by probing plaque lifts with the *PsUGT1* cDNA.

#### RNA Gel Blot and in Situ Hybridization Analysis

Poly(A)<sup>+</sup> mRNA was isolated from root tips excised at different times after induction (as described above). The *PsUGT1* cDNA was <sup>32</sup>P-labeled and used for gel blot analysis (Woo and Hawes, 1997). In situ hybridization was performed as described (Brigham et al., 1998).

#### Hairy Root Transformation and Transgene Constructs

Pea stems were transformed using *Agrobacterium rhizogenes* R1000 to form transgenic hairy roots according to Nicoll et al. (1995) and Wen et al. (1999). Up to 50% of hairy root clones that develop under such conditions are not transformed by the gene of interest, these clones are eliminated by selection on medium supplemented with appropriate antibiotics to select for roots expressing the transgenes of interest (Nicoll et al., 1995). Each root proliferates within days into dozens of branch roots, which can be excised, subcloned, and allowed to proliferate for analysis.

A construct for expressing antisense RNA was made in pBI101 (Clontech, Palo Alto, CA). The *PsUGT1* promoter (-892 to +450) was ligated with the reverse-oriented *PsUGT1* cDNA (+1440 to +470) and inserted into pBI101, replacing the sequence encoding  $\beta$ -glucuronidase. More than twenty separate sets of transformation experiments were performed for each construct of interest (*PsUGT1* promoter-*PsUGT1* antisense cDNA; *PsUGT1* promoter-*PsUGT1* sense cDNA; cauliflower mosaic virus 35S promoter [35S]-antisense *PsUGT1* cDNA; and 35S-*PsUGT1* sense cDNA). For each transformation experiment, 60 to 70 stems were transformed for each construct, for a total of >1000 replicate stem inoculations. For localization of promoter activity, the *PsUGT1* promoter (positions -892 to +450) was inserted into pBI101 expressing *uidA* (encoding  $\beta$ -glucuronidase) as a reporter gene.

#### Alfalfa Transformation and Phenotypic Analysis

Leaf discs from *Medicago sativa* cv Regen were transformed with *A. tumefaciens* strain LBA4404 using the alfalfa transformation and regeneration procedure described previously (Fang and Hirsch, 1998). Hundreds of embryos were obtained for each experiment using control or nonlethal constructs (i.e., *PsUGT1*-sense, 35S-sense, and 35S-antisense), but no embryos were obtained following transformations with lethal constructs (i.e., 35S-diphtheria toxin and *PsUGT1*-antisense). For each experiment, 50 leaf discs were inoculated, and embryos were selected and regenerated on appropriate media. Five independent transformation experiments were performed for each of the test constructs. The transgenic plants were propagated vegetatively. Healthy stems were cut and rooted for 10 to 15 days in a perlite and vermiculite mixture (1:1). The rooted plants were transferred into soil and grown for another 2 to 10 months, yielding hundreds of regenerants for analysis.

Antisense plants were propagated for at least three generations before analysis of phenotype. Sections of 10 to 40  $\mu$ m were cut from embedded tissue and stained with toluidine blue according to Berlyn and Miksche (1976). To measure border cell production, we removed regenerated alfalfa plants from pots and washed the roots to remove potting soil and existing root border cells. The roots were placed onto filter paper, and root tips were examined microscopically to evaluate border cell numbers after 24 hr. More than 70 roots in three independent samples of regenerated control and antisense mRNA plants were examined.

#### Synchronization of Cell Division in Alfalfa Roots and Measurement of Rate of DNA Synthesis

Alfalfa roots of both control and antisense plants were treated with 12 mM 5-aminouracil for 2 hr to synchronize mitosis (Jakob and Trosko, 1965). Roots were washed with water and incubated in 2% glucose. At 3-hr intervals, DNA in roots was pulse-labeled by <sup>3</sup>H-thymidine for 30 min. For each time point, total roots from each of three stem cuttings were collected separately, and the DNA was isolated for measurements of <sup>3</sup>H-thymidine-labeled DNA. The experiment was repeated three times, and values given in Figure 7 represent the mean from all three experiments; standard errors ranged between 8 and 13% of the mean.

#### Protein Gel Blotting Using Antihistidine Antibody

A construct was made in which the *PsUGT1* cDNA was tagged with six histidines at the C terminus. At the 3' end of *PsUGT1* cDNA (+470 to +1510), 18 nucleotides (CATCACCACCATCAC) encoding six histidines were ligated by polymerase chain reaction. The resulting *PsUGT1-6-His* construct was transformed into pea hairy roots under the control of the 35S promoter. The transgenic hairy roots were ground on ice, and the cellular components were size-fractionated by ultracentrifugation at 200,000g for 1 hr into microsomal and cytoplasmic components. Protein was extracted from these components by using 6 M guanidine-HCl. Twenty milligrams of each protein extract was size-fractionated by 10% SDS-PAGE. The proteins were subjected to gel blot analysis by using a commercially available antihistidine antibody (Qiagen).

### Neurospora crassa Transformation

Wild-type *N. crassa* strain 74-OR23-1VA spheroplasts were generated and transformed by described methods (Staben et al., 1989). The *PsUGT1* cDNA under the control of the *cpc-1* promoter in plasmid pMP4 (a gift from M. Plamann, University of Missouri, Kansas City) was cotransformed with pCB1004 (Carroll et al., 1994) into *N. crassa* spheroplasts. Hygromycin-resistant transformed strains were selected. DNA gel blot analysis was performed on the transformants to confirm that the *PsUGT1* sequences had been introduced into them. DNA gel blot analysis was performed on total RNA of the transformed *N. crassa* strains to confirm that *PsUGT1* was being expressed.

### In Vitro Enzyme Assay of Endogenous Pea Glucuronosyltransferase

Microsomes from 200 control root tips and 200 root tips whose border cell development had been induced by removing existing border cells (as described in Plant Growth, above) were isolated by differential ultracentrifugation at several time points after induction. Three replicate sets of 200 root tips were included for each treatment in each experiment. These microsomes were incubated with UDP-<sup>14</sup>C-glucuronic acid for 2 hr at room temperature. The <sup>14</sup>C-glucuronic acid-conjugated reaction products were collected by binding to cellulose. <sup>14</sup>C incorporation was measured by using a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Three independent experiments were performed, and values represent the means of all three experiments. Standard errors were <10% of the mean.

### In Vitro Enzyme Assay of Transformed *N. crassa*

Pea roots were ground and extracted in boiling water for 20 min. After centrifugation, the supernatant was used as a source of an acceptor substrate in an in vitro enzyme reaction. Cellular extracts including microsomes from *N. crassa* transformants were incubated with UDP-<sup>14</sup>C-glucuronic acid, UDP-<sup>14</sup>C-glucose, or UDP-<sup>14</sup>C-galactose as donors and pea tissue extracts. For one negative control, an in vitro enzyme reaction was performed with cellular extracts from untransformed *N. crassa*. For a second negative control, an in vitro enzyme reaction was performed with boiled cellular extracts from *N. crassa* transformants. Reaction products were separated on a silica gel 60 F254 thin-layer chromatography (TLC) plate (Sigma) with 8 volumes of isopropanol, 8 volumes of pyridine, 1 volume of acetic acid, and 4 volumes of water. Activity of microsomes was titrated by assaying the NADPH-cytochrome C reductase (Nega and Grunwaldt, 1997). After separation of reaction products, x-ray film was exposed to the TLC plate at -70°C for 14 to 21 days. The assay was repeated eight times and yielded the same result each time.

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